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Molecular signatures of dissolved organic matter in a tropical karst system

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Abstract

Karst areas are widespread landforms present on all continents, formed by the dissolution of carbonate or evaporite host rock. Little is known about the composition and nature of dissolved organic matter (DOM) as it moves through karst systems, although karst DOM has been recognized as important for a range of natural processes. Microbial communities living in karst systems are some of the most diverse and intriguing on the planet, and their metabolism and life cycle can give clues related to the development of a host of different life forms. Karst areas are also of interest due to their mostly subterranean hydrology, and the repercussions of these processes on local carbon cycles.

We illustrate some of the processes acting on DOM in karst waters through the analysis of soil, drip and cave pool waters at the tropical site of Yok Balum Cave, in southern Belize. Water samples were analyzed using ultra-high resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR-MS), a technique that enables the resolution of single

molecular formulae within a DOM spectrum. We perform multivariate statistics to detect trends in the data and identify provenance of detected molecular components. In addition to karst waters, four aliquots of a powdered stalagmite sample from the same cave system are analyzed. Our results show a clear gradient between the soil and the cave system. We hypothesize that both sorption on mineral surfaces and microbial reworking are responsible for the observed trend in DOM composition. The stalagmite extracts show an anomalous DOM pattern, which may be due to a variety of factors, including microbial activity on the stalagmite surface and different affinities of compounds to incorporation in the carbonate. The goal of this study was to follow the molecular transformations of DOM on its journey from the surface to the cave, and to provide a molecular basis for the establishment of stalagmite DOM proxies in karst systems.

1. Introduction

Karstified carbonate and evaporite lithologies cover about 20% of the Earth's surface and are regarded as sensitive ecosystems and crucial groundwater aquifers (Ford and Williams, 2007). They are characterized by complex, mostly subterranean hydrology with large variability in flow regimes and discharge amounts (Baker et al., 1997). Organic matter from surface vegetation and soil infiltrate the karst system via groundwater in the form of particulate, colloidal, or dissolved organic matter (Simon et al., 2007; Hartland et al., 2012). Particles are effectively removed from the solution by pore blockage in the aquifer (Hartland et al., 2012), except for larger conduits and fractures, where discharge occurs very rapidly (Baker et al., 1997; McCarthy and McKay, 2004). Therefore,

most of the organic matter transported through the karst is in dissolved or colloidal form (Simon et al., 2007; Hartland et al., 2012). The transport and decomposition of organic matter in the vadose zone has been recognized as a potentially important component of the carbon cycle and a foundation for biological activity in karst systems (Simon et al., 2007; Benavente et al., 2010; Noronha et al., 2014; Mattey et al., 2016).

Very little is known about biotic and abiotic karst processes influencing dissolved organic matter (DOM), mainly due to the inaccessibility of karst systems. DOM transported with groundwater plays an important role in a number of karst-related processes, both as a source of nutrients for karst fauna (Simon et al., 2007; Shabarova et al., 2014) and for the transport of trace metals and contaminants (Hartland et al., 2012; Rutlidge et al., 2014). The distinct provenance of the recharge water and the absence of light in karst systems, provides a unique opportunity to study non-photic processes acting on DOM (Birdwell and Engel, 2009). Karst systems, and especially caves, have been the subject of a number of studies on biological activity in the aphotic zone (Shabarova and Pernthaler, 2010; Baskar et al., 2011; Pacton et al., 2013; Shabarova et al., 2014; Tisato et al., 2015). These environments are generally nutrient-limited, and DOM fluxes represent an important source of carbon for karst fauna (Einsiedl et al., 2007; Simon et al., 2007). Microbial decomposition of DOM results in very high CO_2 levels deep in the vadose zone (up to 60,000 uL/L) (Benavente et al., 2010; Mattey et al., 2016), which have been hypothesized to be responsible for large offsets between soil CO_2 radiocarbon ages and radiocarbon bomb-spikes observed in speleothems (Noronha et al., 2015).

Much of the past efforts in studying the composition and provenance of DOM in karst systems revolved around the question of its usefulness as speleothem paleoclimate proxy (e.g., Blyth et al., 2016, and references therein). Several studies have focused on the fluorescent properties of organic carbon in cave drip waters and stalagmites, as this parameter is easily measured and provides information on past environmental conditions and rainfall at the cave site (e.g., Baker, 1998; McGarry and Baker, 2000; Hartland et al., 2010; Quiers et al., 2015). Other studies presented methods for the extraction and characterization of bulk organic carbon (Blyth et al., 2013a, 2013b; Li et al., 2014) or specific molecular compounds (Xie et al., 2003; Huang et al., 2008; Blyth and Watson, 2009; Blyth et al., 2011; Blyth and Schouten, 2013; Bosle et al., 2014) from stalagmites. However, low organic matter concentrations and large uncertainties with respect to the provenience and environmental significance of these compounds hamper their routine-based application to stalagmites (Wynn and Brocks, 2014; Blyth et al., 2016). These studies illustrate the transport and alteration of organic matter in karst systems and its incorporation into speleothem carbonate. Both leaching of organic matter from the soil, as well as in-situ microbial production have been recognized as contributions to the cave/stalagmite DOM spectrum (Birdwell and Engel, 2009; Shen et al., 2015). The amount of soil-derived organic matter being leached into the vadose zone is related to the amount of rainfall and the flow path, with longer residence times leading to increased DOM processing through physicochemical and microbial processes, and therefore to an increased loss of the soil DOM fingerprint (Blyth et al., 2016). However, the focus of these studies on single compound classes or sizes precludes a comprehensive portrayal of the molecular characteristics of DOM in karst systems, and open

questions remain with respect to the processes occurring at the interface between the soil/vadose zone and cave environment, as well as to the incorporation of DOM in stalagmites.

With this study, we characterize the molecular transformation of DOM on its route from the terrestrial surface to the cave environment, and explore the potential of stalagmite DOM as a tracer for information on past land cover. We present results from the tropical Yok Balum Cave system, located in Belize, Central America. Water samples from soil, and cave drips and pools, as well as extracts from one stalagmite were analyzed in detail using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR-MS). Applying multivariate statistical methods to the mass spectral data, we detect significant differences between surface and cave samples, suggesting substantial reworking of DOM during transport through the karst.

2. Study site

Yok Balum Cave is located in the Toledo district in southern Belize, Central America (16°12'30.780'' N, 89°4'24.420'' W, 336 m above sea level; Fig. 1). The cave developed in an isolated hill part of a SW-to-NE trending karst ridge, with steep hillsides covered in dense jungle vegetation. Although the surrounding valley and lower hills have been subject to intensive human activity (farming and logging) for at least 2500 years (Culleton, 2012; Walsh et al., 2014; Prufer et al., 2017), it is likely that the inaccessible hilltop of Yok Balum Cave remained mostly unaffected by farming or intensive logging, and thus reflects anthropogenically undisturbed environmental conditions. The soil above Yok Balum Cave can be classified as a relatively thin leptosol (*WRB 2006*), extending

more than 30 cm deep only in isolated pockets. In profile, the soil shows no clearly distinguishable horizons, except for a thick humus layer at the top ~5 cm and increasing contributions from the carbonate host rock at the bottom (Lechleitner et al., 2016).

Climate in southern Belize is governed by the annual migration of the Intertropical Convergence Zone, with clear seasonality in precipitation: boreal summer months experience abundant rainfall (up to 700 mm), whereas winters are dry with a negative water balance (evapotranspiration > precipitation) (Poveda et al., 2006). Air temperature is relatively stable throughout the year, with a seasonal difference of only ~4 °C between summers and winters.

Yok Balum Cave is a ~540 m-long trunk passage (Ridley et al., 2015b, Fig. 1), richly decorated with speleothems. In-depth cave microclimate monitoring since 2011 revealed a strongly seasonal and diurnal ventilation regime driven by thermally induced air density differences between cave and surroundings (Ridley et al., 2015b), resulting in pCO_2 levels between 419 (January) and 507 (June) ppmv (Kennett et al., 2012). The cave temperature is stable throughout the year at 22.3 ± 0.5 °C (Ridley et al., 2015a), and relative humidity is high (>95%) (Kennett et al., 2012). The drip sites investigated in this study are generally active all year long, but throughflow is reduced during the dry season months (Ridley et al., 2015a). Both pore flow and fracture flow drips can be identified in the cave, promoted by the presence of a doline above the study site, with focused recharge and deeper soil (Ridley et al., 2015a). High-resolution stable oxygen and carbon isotope ratio records from two stalagmites (YOK-I and YOK-G) have provided detailed information on past climatic and environmental conditions (Kennett et al., 2012; Ridley et al., 2015a). Past changes in karst

hydrology and carbon cycle dynamics were studied by combining stalagmite and soil ^{14}C measurements, and modeling (Lechleitner et al., 2016). A very young soil organic matter (SOM) spectrum suggests rapid soil turnover, which is reflected in pronounced stalagmite ^{14}C bomb spikes in the cave. Overall, the influence of hydrological conditions in the karst, especially the degree of open vs. closed system conditions, appears to dominate the reservoir effect ("dead carbon fraction") in Yok Balum Cave stalagmites (Lechleitner et al., 2016). The undisturbed location and small catchment size of Yok Balum Cave, combined with the wealth of information available on the hydrology of the karst system and the cave's microclimate, make it an excellent site for research on DOM transport and compositional evolution from surface to cave.

3. Methods

3.1. Field sampling and DOC analysis

Water samples were collected in pre-combusted amber glass bottles previously spiked with 85% H_3PO_4 (puriss. grade). Drip and pool water samples were collected during two field campaigns in June 2013 and 2014. We distinguish between slow, seepage flow drips (YB_DW_1 to 4 from 2013, and 4, 6, 7, and B-day old from 2014), and fast, fracture flow drips (B1 and B2, Table 1, Fig. 1C). Samples from the slow drip sites were collected by leaving the bottle under the drip for a period of hours to several months (in the case of B-day old), depending on the drip rate. Copious water discharge at the fast drip sites allowed on the spot sample collection. Pool waters were filtered at the sampling site using pre-combusted 47 mm GF/F filters (Whatman™). Soil water samples were collected using two silicium carbide pore water samplers (SIC20, UMS) installed in the soil

profile at ~30 cm depth. Samples were collected approximately every two weeks over the entire rainy season (July-November 2014). Upon arrival in the laboratory, the samples were filtered through pre-combusted 47 mm GF/F filters (Whatman™), acidified to pH 2 using 85% H_3PO_4 (puriss. grade), and stored in cool, dark conditions until analysis. A sample of ultrapure water was filtered as well, to quantify the procedural blank.

Dissolved organic carbon (DOC) concentrations of all water samples were determined on a total organic carbon analyzer (TOCL Shimazu) equipped with an ASI-L autosampler at the Institute of Terrestrial Ecosystems, Department of Environmental System Sciences at ETH Zurich. Before analysis, 10 ml aliquots of the samples were filtered using 0.45 μm PTFE filters to remove particles, and placed in a vacuum chamber for 5-10 min to remove excess inorganic carbon from the solution. Measurement quality was ensured using a Certipur 1000 mg/L Total Organic Carbon (TOC) standard (Merck) with a concentration of 20 ppm.

3.2. Stalagmite extracts

Stalagmite YOK-K is about 20 cm long and was collected from Yok Balum Cave in 2013. The samples used for FT-ICR-MS stem from the stalagmite base, with an estimated age of ~2000 years, and were chosen because of their dense and clean primary aragonite mineralogy (Suppl. Fig. 1). A ~5 g aliquot of stalagmite was leached in 1N HCl to remove external contamination from sample collection and handling, rinsed in ultrapure water and dried at 60 °C overnight. The sample was then powdered and homogenized using an agate mortar. For the FT analysis, four aliquots of 400-500 mg $CaCO_3$ were transferred to 12 ml borosilicate

Exetainer vials (Labco, High Wycombe, UK), and dissolved using 1.5 ml 85% H_3PO_4 (puriss. grade) and 3.5 ml of ultrapure water. After complete $CaCO_3$ dissolution, the samples were subjected to a gentle vacuum for ~1 hour using a rotary evaporator. This procedure is very similar to the method by Blyth et al. (2013a and 2013b), as it was found that without vacuum treatment the removal of inorganic carbon from the solution was insufficient, masking the DOM signal. Three additional aliquots of the carbonate powder were dissolved and oxidized using wet chemical oxidation following Lang et al. (2016), and their TOC content was determined using irMS.

3.3. ESI-FT-ICR-MS

DOM was extracted from 39 previously acidified (pH 2) samples using 100 mg Bond-Elut PPL cartridges (Agilent Technologies) (Dittmar et al., 2008). An aliquot of ultrapure water was subjected to the same protocol as a procedural blank. The cartridges were soaked overnight in LC-MS grade methanol, then rinsed twice with ultrapure water, methanol and 0.01N HCl before processing the samples. The volume of sample passed over the cartridge was adjusted according to the sample DOC amount to obtain an amount of 20 µmol for FT-ICR-MS measurement. Following the sample, the cartridge was rinsed twice with 0.01N HCl and then dried with ultrapure He gas. The DOM retained on the cartridge was eluted using LC-MS Chromasolv grade methanol (Sigma Aldrich). Extracts were diluted with ultrapure water according to their concentration to obtain 10 ppm of DOC, and filtered using 0.2 µm PTFE syringe filters before analysis by FT-ICR-MS.

Electrospray ionization produced negative ions of the DOM extracts, which were analysed on a 15 T ultrahigh-resolution FT-ICR-MS (Bruker Daltonics, Bremen, Germany) at the University of Oldenburg, Germany. Samples were measured in two batches, separated by about 11 months. In order to test the reproducibility of the measurements, a few samples were measured in both batches.

Mass spectra for each sample were collected in 500 scans and calibrated against a list of marine DOM ($\sigma < 0.05$), and the method detection limit (MDL) was calculated following Riedel and Dittmar (2014). Blank correction was based on the signal-to-noise (s/n) ratio, where the noise in the dataset is defined by the MDL. All blank peaks with s/n >20 were excluded from further analysis. Blank correction was carried out differently depending on the type of sample. All water samples were corrected using both the filtering and cartridge blanks, whereas the stalagmite extracts were corrected using a procedural blank extract and the cartridge blank. Molecular formulae were assigned to peaks following the criteria for elemental combinations defined by Koch et al. (2007). Formulae not matching the criteria or only present in one sample were excluded from the final dataset. Double assignments (peaks with more than one possible formula) were treated following the criteria published in Seidel et al. (2014) and the formula less likely present in DOM was excluded whenever possible. A key parameter for the assignment of molecular formulae is the modified aromaticity index (AI_{mod}) (Koch and Dittmar, 2006), which indicates the presence of aromatic and condensed aromatic compounds. Assignment of molecular formulae to subgroups was determined via their stoichiometry after Seidel et al. (2014), based on the AI_{mod}, established molar ratios, and heteroatom contents. We identify the following compound groups (as described in Seidel et al., 2014): (1)

polycyclic aromatics (PCAs, AI_{mod} > 0.66), (2) highly aromatic hydrocarbons, including polyphenols and PCAs with aliphatic chains (0.66≥ AI_{mod}> 0.50), (3) highly unsaturated compounds, including phenols for example from lignin degradation from soils (AI_{mod} ≤ 0.50 and H/C < 1.5), (4) unsaturated aliphatic compounds (2.0 > H/C ≥ 1.5), (5) saturated compounds, including fatty and sulfonic acids, and carbohydrates (H/C ≥ 2.0, N > 0). Separations according to the heteroatom content result in the following groups: (7) molecular formulae containing N, (8) molecular formulae containing S, (9) molecular formulae containing P, (10) formulae containing N and S, and (11) molecular formulae containing N and P (Seidel et al., 2014). It is important to note that there are likely multiple isomers behind each molecular formula, and therefore the assigned molecular groups are only suggestions for likely structures. They should not be interpreted as unambiguous structure assignments.

3.4. Statistical analysis

After normalization of the peaks to total signal intensity, Principal Component Analysis (PCA, Matlab) was used to visualize the dataset, and to identify and exclude outlier samples and formulae. A total of 6 samples (one from a fast drip site, three from slow drip sites, and two soil water samples) were found to be strongly skewing the PCA. After closer inspection, these samples showed strong signs of contamination in their spectra (see Suppl. Fig. 2, for an example), low numbers of molecular formulae (between 20 and 80% less than the average of the corresponding sample group), and in some cases very high DOC values (> 50 mg/L). Blank assessment during all laboratory procedures showed no large contamination sources, and it is likely that the contamination happened during

sample collection in the field. Therefore, these samples were excluded from further analysis.

For multivariate statistical analysis, only the 3661 most abundant peaks in each sample were considered. We applied non-metric multidimensional scaling (NMDS, R, package vegan; Oksanen, 2008) to study the differences between individual sample groups (soil, drip, and pool waters, and stalagmite extracts). We found that NMDS appears to be more robust than PCA for our dataset, as it relies on ranked orders rather than linear relationships, which can be useful for datasets affected by contaminants and/or analytical shifts. Given the non-selective nature of FT-ICR-MS and the challenges in instrumental stability, both considerations are valid for this analytical approach.

4. Results

Measured DOC concentrations in all water samples ranged between 2.65 and 54.4 mg/L (average: 10.8 mg/L), with a tendency for slightly higher DOC amounts in the soil samples, compared to the drip and pool waters (Table 1). TOC concentrations determined on three aliquots of stalagmite YOK-K average at 0.14 mg C/g stalagmite, in range with previously published stalagmite TOC values (e.g., 0.11 mg/g in Blyth et al., 2013a, 0.12-0.14 mg/g in Li et al., 2014, 0.27-3.03 mg/g in Quiers et al., 2015).

After ESI-FT-ICR-MS, 14311 mass peaks between m/z= 154 and 999 were detected in the entire dataset. The number of molecular formulae present in the individual samples, however, is highly variable between 3661 and 7981 formulae per sample (after blank correction). On average, soil water samples contain the most molecular formulae (6182, σ = 1058), followed by pool waters

(4975, σ = 962), slow drip waters (4840, σ = 1010), fast drip waters (4833, σ = 1050), and stalagmites (4255, σ = 396). Only 629 molecular formulae are ubiquitously present in all samples, highlighting the large differences between sample groups (Suppl. Fig. 3). Within sample groups the percentage of formulae present in all samples is much higher: drip waters on average contain 43.3% formulae specific for their group, soil waters 52.49%, and pool waters 64.98%. The stalagmite samples share even 80.69% of their formulae, however, this is due to the fact that the four extracts are deriving from the same stalagmite section after homogenization.

NMDS performed on the 3661 most abundant formulae per sample shows a clear separation between sample groups. Soil, pool and drip waters are ordered along a line in NMDS 2 vs. NMDS 1 space (Fig. 2). Stalagmite extracts fall within the same range of NMDS 2 as the drip waters, but are far removed from all water samples along the NMDS 1 axis.

Comparing magnitude-weighted relative abundances of compounds in the samples yields insight into molecular differences depicted by NMDS (Suppl. Table 1, Fig. 3). All samples are particularly rich in highly unsaturated compounds (all ~40%), with important additional contributions from polyphenols and unsaturated aliphatics (both < 10%). Smaller contributions stem from combustion-derived (pyrogenic) condensed aromatic compounds, peptides, sugars and fatty acids. A clear gradient exists between soil and drip waters in oxygen-rich highly unsaturated and polyphenolic compounds, as well as black carbon, with drip waters being practically devoid of these compounds. On the other hand, drip waters are enriched in peptides, sugars and fatty acids, which are present in very low amounts in soil water samples. This trend is

particularly clear in the slow drip water samples, whereas the fast drip water samples tend to be more similar to the soil water samples. The composition of the pool water samples appears to be overall more similar to soil waters than drip waters, which might be related to a dominant fracture-flow source. Stalagmite extracts are very different from the other sample groups, with very high relative abundances of highly unsaturated, polyphenolic, black carbon and sugar contributions (Suppl. Table 1, Fig. 3).

5. Discussion

5.1. Surface-to-cave gradient

Statistical treatment of the dataset using NMDS reveals a clear gradient between surface soil and cave waters, with increasing compositional dissimilarity between soil, pool, and fast and slow drip waters (Fig. 2). These substantial differences in the molecular composition of soil and cave waters are also visible in the raw dataset (Suppl. Fig. 3), and suggest substantial reworking of DOM between surface and cave.

Soil waters are most abundant in highly unsaturated (~40%), polyphenolic (~6%) and unsaturated aliphatic (~4-6%) compounds. These compounds typically derive from vascular plants, which constitute the precursors and sources for overwhelming majority of SOM in the topsoil (Trumbore, 2009), whereas the deeper soil might be more strongly affected by microbial reworking processes (Rumpel and Kögel-Knabner, 2011). This is consistent with the fact that the soil above Yok Balum Cave is very thin (< 30 cm), likely resulting in a less important microbial reworking component. Slow seepage water fed drips in Yok Balum Cave show a substantially larger contribution from microbially-

derived compounds, such as peptides (up to 16% relative abundance), sugars, and saturated fatty acids. Although the overall contribution of highly unsaturated and polyphenolic compounds is very similar to the soil waters, slow drips are depleted in oxygen-rich compounds, as well as in pyrogenic molecules (Fig. 3). In contrast, water sampled from the two fast fracture flow drips and from the pools is characterized by a larger component of allochtonous, soil-derived DOM, evident from their closer proximity to the soil water samples (Fig. 2).

Generally, a trend towards more deoxygenated and hydrogenated molecules (lower O/C and higher H/C in the Van Krevelen diagram) is visible between soil and drip waters, highlighting the aforementioned compositional changes between sample groups (Fig. 4). These trends are very well reproduced when color-coding the individual formulae with their NMDS 2 site value (Fig. 4), and highlight the importance of the process described by NMDS 2 in the water samples.

From the surface, DOM enters the karst system principally via the soil system. SOM is a complex mixture of both plant-derived and microbial components, where root respiration and microbial decomposition are the dominant reworking processes (Trumbore, 2009). It is becoming increasingly evident that SOM decomposition is highly dependent on its accessibility to decomposing microorganisms, and that sorption of organic compounds to minerals can protect and stabilize significant portions of the SOM spectrum (Lehmann and Kleber, 2015). The progressive trend in deoxygenation and enrichment in microbially-derived compounds found at Yok Balum Cave can be embedded into this context, and validates the emerging view of DOM in caves and stalagmites being derived from both the soil, as well as from autochtonous microbial communities (Blyth et

al., 2016). DOM is subject to reprocessing both by biological degradation, as well as through physicochemical adsorption to minerals during its transfer through the soil and vadose zone (Shen et al., 2015; Mattey et al., 2016), both of which become increasingly important with longer residence time of the water in the karst (Shabarova et al., 2014; Blyth et al., 2016). Drip waters originating from high discharge and fracture flow conduits are therefore likely to retain the original soil DOM fingerprint, whereas seepage flow waters will have experienced a higher degree of reprocessing. Significant subsurface reworking of DOM in karst systems has been observed previously, even suggesting only minor contributions of surface components to cave water DOM (Einsiedl et al., 2007; Simon et al., 2007; Birdwell and Engel, 2009). A more recent estimate proposes a microbial origin of 15-34% of groundwater DOM, and a removal of about 90% of surface-derived DOM before reaching the saturated zone (Shen et al., 2015).

The clear separation between sample types is also detected by Kendrick Mass defect (KMD) analysis, a 2D-visualisation of mass spectra that allows the detection of possible structural relationships (i.e., homologous series) (Hughey et al., 2001) (Suppl. Fig. 4). Very similar observations with respect to KMD values were made by Einsiedl et al. (2007) for karst waters, as well as by results from DOM-coagulation experiments, where compounds with lower KMD values were selectively removed from the solution after addition of Al or Fe (Riedel et al., 2012). Indeed, selective removal of more oxygenated molecules by Fe(III)-induced flocculation has been observed in rewetted fens, indicating that redox interfaces in natural waters can play a key role in the reworking of DOM (Riedel et al., 2013).

Our data suggests that both abiotic and biotic reactions play an important role in the alteration of DOM in the vadose zone at Yok Balum Cave. Abiotic reactions may include sorption and desorption of DOM from the karst mineral matrix, which has shown to be a reversible process in karst aquifers (Jin and Zimmerman, 2010). Preferential removal of oxygenated molecules at redox interfaces is a likely additional abiotic process influencing the karst water DOM spectrum. Complexation of DOM with trace metals has been demonstrated (Hartland et al., 2012; Rutlidge et al., 2014), and appears to be the dominant process carrying transition metals from the surface to the cave (Hartland et al., 2011). Sorption-desorption processes in the karst could play an important role in the effectiveness and rate of transport of soil-derived trace metals and contaminants. Finally, microbial alteration of DOM in the vadose zone appears to be an important contributor, especially for slower drip waters, where longer residence time allows for stronger microbial transformation of the DOM. In addition, slightly decreasing DOC concentrations between surface and cave waters suggest that a (small) portion of DOM is likely also removed during transport and stored in the karst or oxidized.

5.2. Relevance for stalagmite DOM proxies

Over the past decades, a small but growing body of research has focused on the development of paleoclimate proxies from organic carbon entrapped in stalagmites (e.g., Xie et al., 2003; Blyth and Watson, 2009; Blyth et al., 2011, 2013b; Blyth and Schouten, 2013; Bosle et al., 2014; Li et al., 2014; Quiers et al., 2015). Although with some promising results, questions relating to the

provenance and environmental significance of stalagmite organic carbon still hamper our understanding and the application of these proxies.

In this study, we analyzed extracts from stalagmite YOK-K to investigate on the extractable stalagmite DOM component and compare it to water samples from the karst system. Our data displays a very different molecular fingerprint for stalagmite DOM, compared to that of drip and soil waters (Suppl. Fig. 3). In the NMDS, stalagmite samples are isolated from the other sample groups along the NMDS 1 axis, but are in the range of the slow drip waters along the NMDS 2 axis (Fig. 2). Stalagmite samples have very low H/C ratios, comparable to the soil samples, and cover a wide range of O/C ratios (Fig. 4). The anomalous DOM composition of stalagmite extracts becomes even more apparent when considering relative abundances of the compounds present. Stalagmite extracts have the highest relative contributions of highly unsaturated oxygen-rich, polyphenolic and pyrogenic compounds, reflecting and exaggerating the trend of the soil samples. On the other hand, the extracts also contain large amounts of sugars and peptides, similarly to the slow drip waters.

It is possible that the anomalous molecular composition of stalagmite DOM is related to sample contamination, either during collection, storage or laboratory procedures (Wynn and Brocks, 2014). The thorough cleaning protocol applied to stalagmite samples in this study, and the fact that a wide range of compound groups was analyzed (reducing the risk of misinterpretation of single contamination-derived compound groups), makes intrinsic contamination unlikely. Another possibility is that the stalagmite DOM signature is an artifact associated with carbonate removal and subsequent degassing of samples under

vacuum, for example resulting in the selective removal of certain compound classes in favor of others.

It is also possible that the process of incorporation of DOM into stalagmite carbonate matrix itself is selective, resulting in discrimination against certain compound types, due to physical and/or chemical properties. Most organic matter in stalagmites is incorporated either between crystals or in pores of the carbonate fabric through mineral adsorption or uptake in fluid inclusions (Ramseyer et al., 1997; Chalmin et al., 2013). Physical and chemical characteristics of molecular compounds, such as size, structure, charge, and polarity are likely to influence their affinity to being incorporated into the stalagmite fabric. Laboratory experiments under controlled conditions indicate rapid and mostly irreversible adsorption of humic acid on calcite (Lee et al., 2005), via weak OH bonds (Chalmin et al., 2013).

Additionally, it should be noted that the comparison between karst water samples, collected over a time span of minutes to months, and extracts from stalagmite YOK-K, which likely average over years to centuries (due to the large sample size needed), introduces a significant temporal sampling bias. Although climatic conditions in Belize were relatively stable over the past two millennia, it is possible that "momentary" present-day soil and drip water samples are not capturing the entire DOM spectrum reaching the cave over long time periods or at a specific time in the past. Further studies focusing on the investigation of stalagmite DOM spectra over time are needed to inform on the temporal variability of incorporated organic matter.

If the DOM signature in stalagmite YOK-K is indigenous, then the process resulting in the alteration of stalagmite DOM compared to drip water DOM

requires consideration. Karst microbial communities are known to use water films in caves as nutrient substrates, and some species have found to be associated with calcite formation or destruction due to their metabolism (Simon et al., 2007; Jones, 2010; Pacton et al., 2013; Tisato et al., 2015). Analysis of glycerol dialkyl glycerol tetraethers (GDGTs) showed substantially different distributions in speleothems and the associated soils, suggesting that GDGTs measured in stalagmites are produced in-situ or in the karst (Blyth et al., 2014; Baker et al., 2016). Strong overprint of the stalagmite DOM signal by in-situ microbial communities could explain the high contribution of sugars in the stalagmite samples from Yok Balum Cave (Fig. 3). The high relative abundance of pyrogenic compounds in stalagmite DOM, on the other hand, could be derived from seasonal burning of fields for agriculture (slash-and-burn), common in the area around Yok Balum Cave. The large amounts of smoke and ash produced by this practice likely accumulate in the soil, due to their recalcitrance to degradation, and could then be transported into the cave (Dittmar et al., 2012).

The question remains as to whether this signature carries interpretable information about climatic or environmental change at the cave site. Although the DOM spectra between stalagmite and soil waters are very different, the stalagmite appears to retain some information from the surface environment (e.g., the total concentration of unsaturated and polyphenolic compounds), which could be used to reconstruct changes in vegetation type and cover. Further studies on stalagmite organic matter are needed to corroborate these results, and to fully test the potential of DOM fingerprinting as a paleo-environmental proxy.

6. Conclusions

We present results from a compositional study of DOM following a hydrological pathway from overlying soils, through a karst network, to Yok Balum Cave in Belize, tropical Central America. Soil, drip and cave pool waters, together with extracts from stalagmite YOK-K from the same cave were analyzed by ultra-high resolution FT-ICR-MS. Multivariate statistical analysis of the data reveals a clear surface-to-cave gradient, shedding light on possible processes driving these changes. On one hand, selective removal of oxygenated unsaturated and polyphenolic compounds is attributed to abiotic processes, such as sorption on the karst mineral matrix, transformation at redox interfaces. On the other hand, increasing abundances of peptides, sugars, and saturated fatty acids in drip waters suggest microbial reworking of DOM in the karst environment. Organic extracts from a stalagmite from Yok Balum Cave exhibit markedly different DOM spectra. Uncertainties with respect to organic matter provenance and methodological artifacts prevent us from drawing concrete conclusions with respect to the applicability of stalagmite DOM as paleo-environmental proxy. However, this method provides a means to simultaneously study thousands of compounds, and characterize speleothem organic matter in unprecedented detail. Our results provide strong motivation for further, in-depth, studies of DOM in karst systems in order to explore the rich molecular archive hidden in DOM.

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Tables:

Table 1: Summary of sample types and DOC content from Yok Balum Cave.

Figures

Fig.1: A - Map of the broader study area. B – Picture of the soil profile taken above the cave. C –Yok Balum Cave map with indications of sampling locations for the soil water, drip and pool water. Soil waters were collected at the surface above the green shaded area. Stalagmite YOK-K was found lying on the ground, and its original location cannot be reconstructed.

Fig. 2: Results for all samples from NMDS. A clear trend both in NMDS1 and NMD2 can be discerned between drip, pool and soil waters, highlighting a gradient between surface and cave. Stalagmite extracts are distinct from the other samples in NMDS1, but have similar NMDS2 as the slow drip waters.

Fig. 3: Relative abundances of selected main compound classes apparent in the samples from Yok Balum Cave. Each column represents a sample, grouped by sample type (stalagmites, drip water, pool water, soil water). Drip waters are separated into slow drips (A), and fast drips (B). Compounds listed (from top left): highly unsaturated oxygen-rich/oxygen-poor, saturated fatty acids with/without heteroatom (-X), polyphenols oxygen-rich/oxygen-poor, sugars with/without heteroatom (-X), black carbon with/without heteroatom (-X) and smaller/larger than C15, peptides.

Fig. 4.: A – Van Krevelen (H/C vs. O/C) plot. Each dot represents one molecular formula unique to one sample group (i.e. the compound is not present in the other groups). B – Van Krevelen diagrams with molecular formulae color-coded based on their NMDS1 and NMDS2 value. The highest NMDS1 values are grouped

at the same H/C vs. O/C values as the stalagmite samples in A. On the other hand, the grouping of NMDS2 values reflects more closely the distribution of soil and drip water samples in A, i.e. drip waters have higher H/C and lower O/C than soil waters. C – Conceptual diagram of the underlying processes responsible for the variability in the Van Krevelen diagrams. Higher H/C ratios indicate more hydrogenated compounds, whereas low O/C ratios suggest deoxygenation. The gradient between surface and cave samples reflects a mixture of these two processes.

Supplementary information:

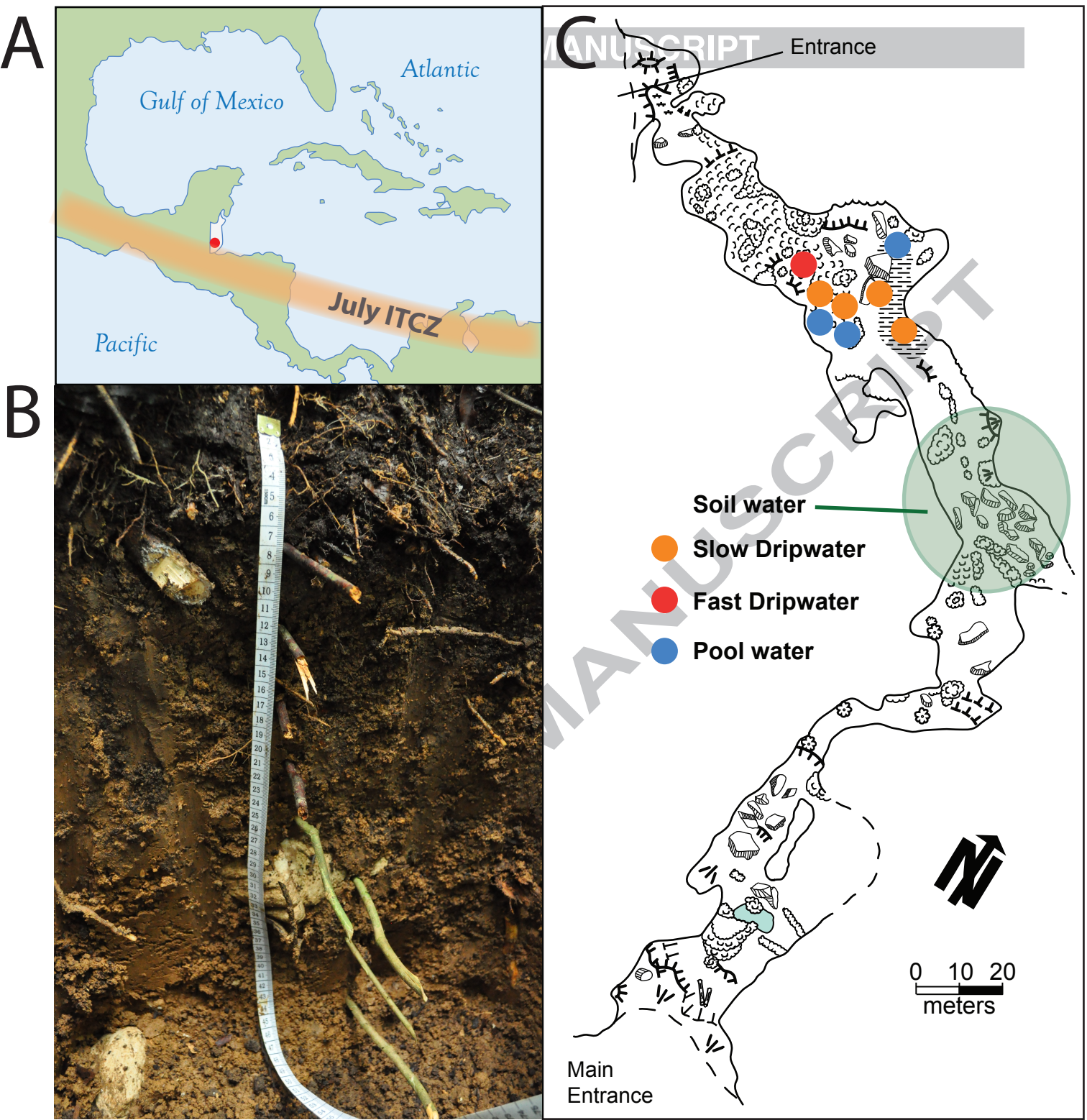
Suppl. Fig. 1.: Photograph of stalagmite YOK-K. A pound coin (22.5 mm diameter) is shown for scale. The insert shows a cut section at the stalagmite base.

Suppl. Fig. 2.: Examples of DOM spectra measured in samples from Yok Balum Cave. Sample DW6 had to be excluded from the final results, due to strong contamination, visible in the raw FT-ICR-MS data.

Suppl. Fig. 3.: Van Krevelen plots of the three end members (soil water, drip water, and stalagmite extracts) color-coded by their signal intensities. The molecular differences between sample groups become apparent.

Suppl. Fig. 4.: CH_2-based Kendrick Mass Defect (KMD) plot, color-coded by sample group.

Suppl. Table 1: Summed weighted relative contributions of compound groups to samples.


A
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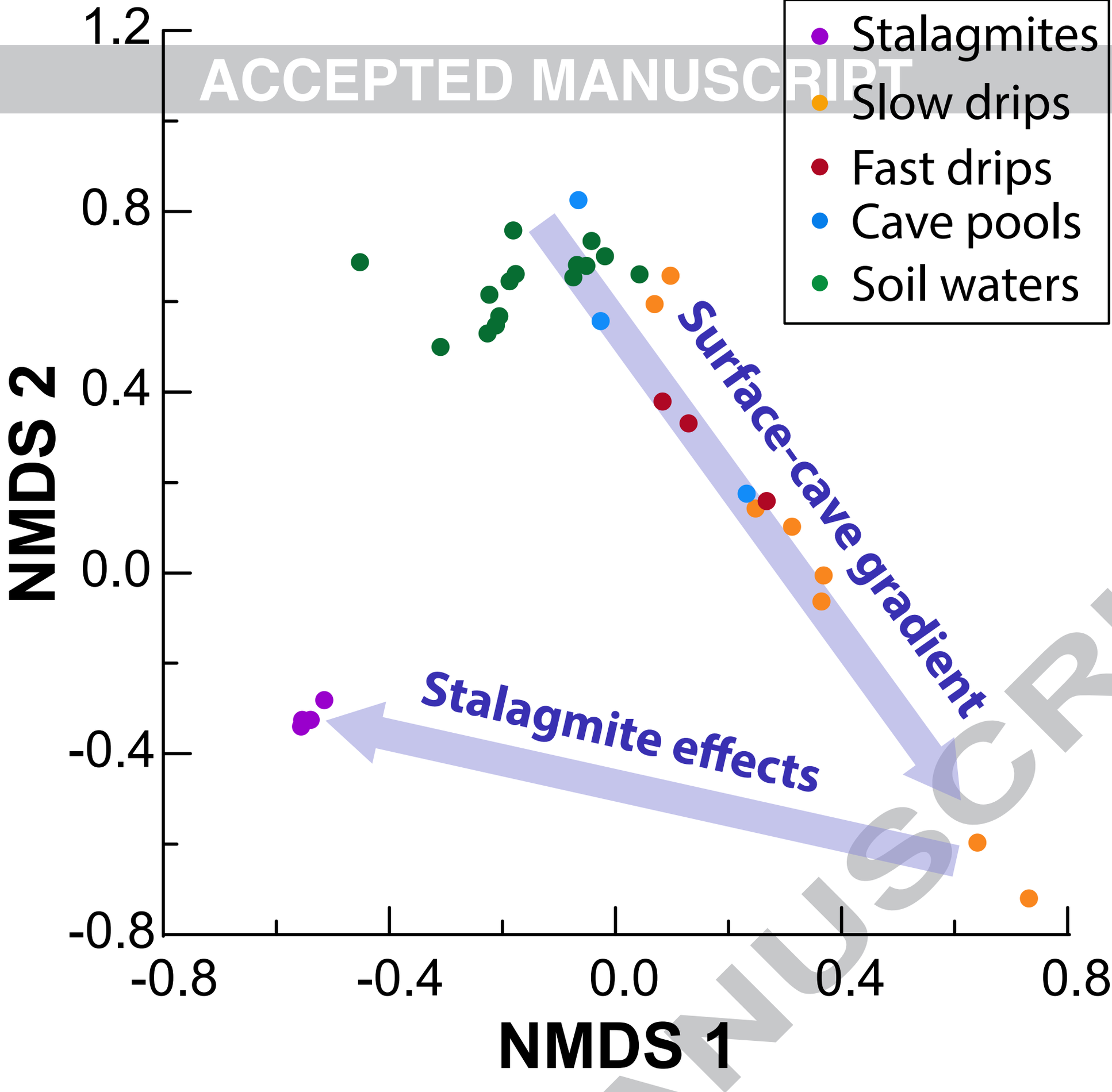
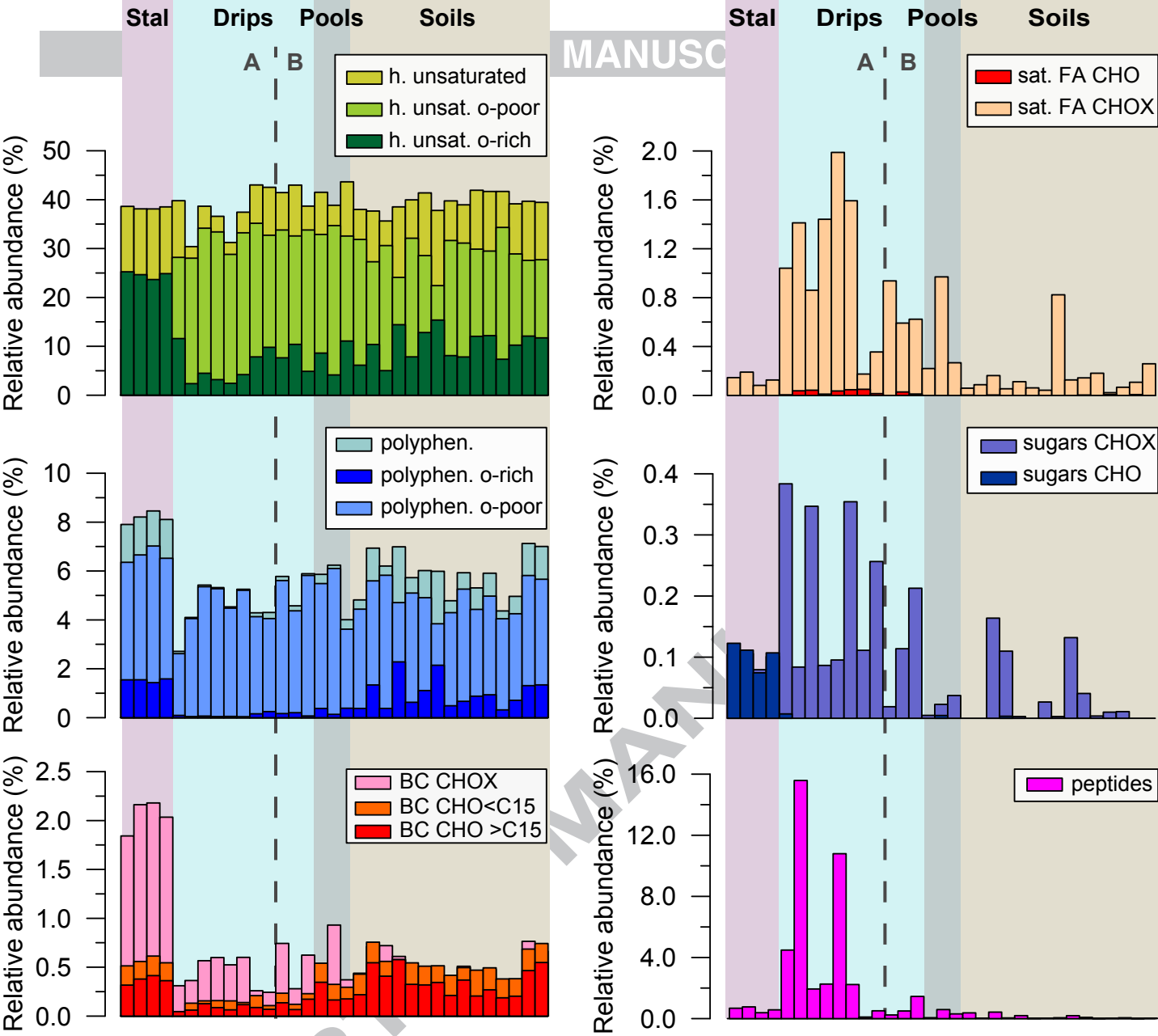
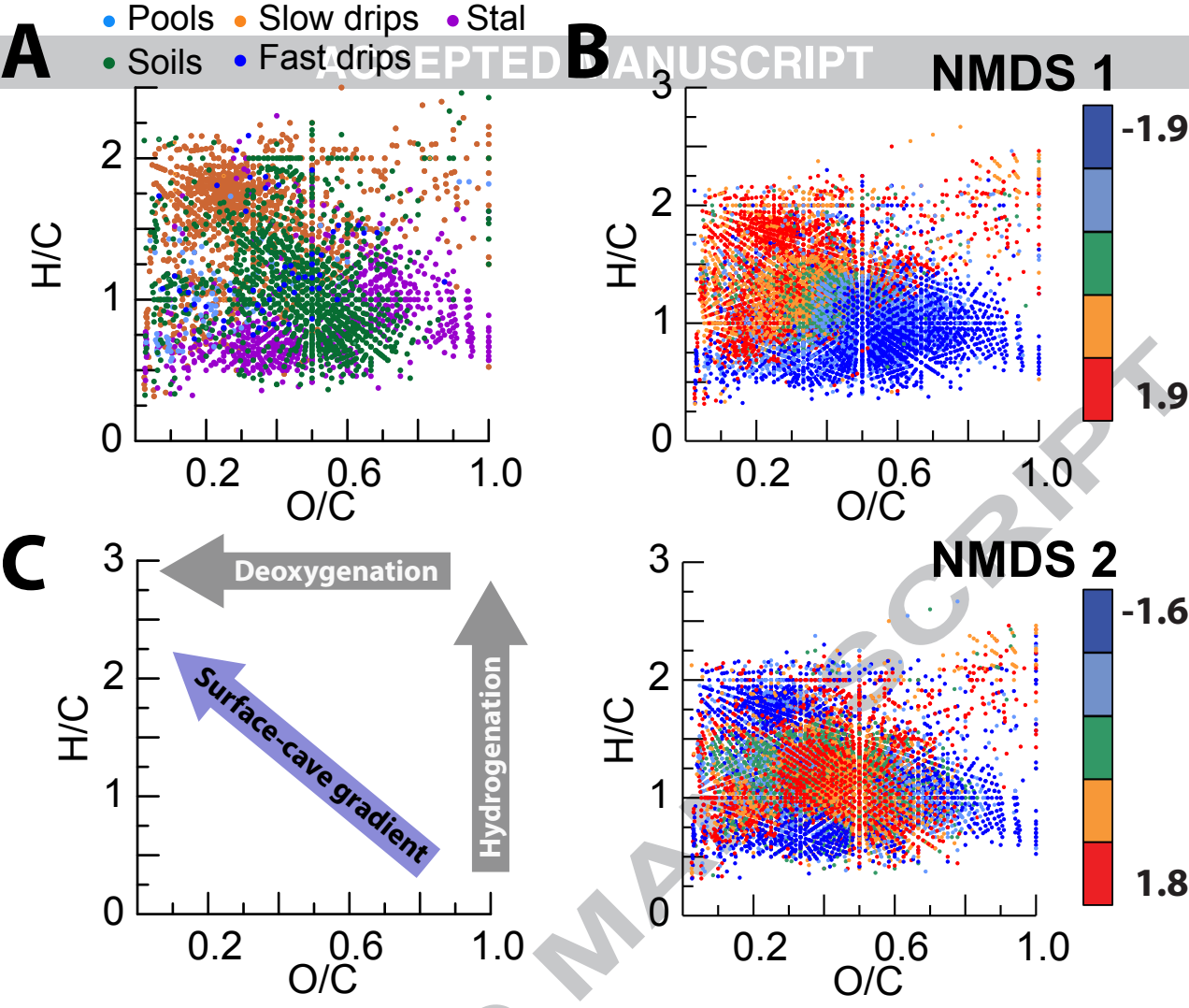



Stal
Drips
Pools
Soils
A
B
h. unsaturated
h. unsat. o-poor
h. unsat. o-rich
Relative abundance (%)
sat. FA CHO
sat. FA CHOX
polyphen.
polyphen. o-rich
polyphen. o-poor
sugars CHOX
sugars CHO
BC CHOX
BC CHO<C15
BC CHO >C15
peptides



Sample name	Sampling year	description	DOC amount mg/L
YB_DW_1 2013	2013	drip water	6.78
YB_DW_2 2013	2013	drip water	3.00
YB_DW_3 2013	2013	drip water	2.36
YB_DW_4 2013	2013	drip water	3.72
YB_DW_4 2014	2014	drip water	2.67
YB_DW_6 2014	2014	drip water	27.90
YB_DW_7 2014	2014	drip water	2.65
B-day old	2014	drip water	3.06
B1	2014	fast drip	54.40
B2	2014	fast drip	2.77
Pool 1	2014	pool water	3.11
Pool 2	2014	pool water	27.40
Pool 3	2014	pool water	3.08
YB_SW_1	2014	soil water	40.90
YB_SW_2a	2014	soil water	3.42
YB_SW_2b	2014	soil water	5.03
YB_SW_3a	2014	soil water	5.29
YB_SW_3b	2014	soil water	4.96
YB_SW_4a	2014	soil water	3.92
YB_SW_4b	2014	soil water	5.04
YB_SW_5a	2014	soil water	47.60
YB_SW_5b	2014	soil water	4.96
YB_SW_6a	2014	soil water	4.00
YB_SW_6b	2014	soil water	4.51
YB_SW_7	2014	soil water	3.63
YB_SW_8	2014	soil water	4.28

Highlights:

- Ultra-high resolution analysis of DOM in karst systems using ESI-FT-ICR-MS
- Clear gradient between soil and cave, influence of biotic and abiotic processes
- Stalagmite DOM spectra retain part of the soil DOM fingerprint
- Important in-situ molecular components from microorganisms